An *In Vitro* Model of Pancreatic Carcinoma

Morphology and In Vivo *Growth*

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Pancreas rudiments from 13-day rat embryos were cultured in the presence of dimethylnitrosamine (DMN) for up to 10 weeks. Pancreas morphogenesis and differentiation occurred during the first week of culture. Acinar cell degeneration and necrosis began on the fifth day of culture and resulted in almost complete loss of acinar cells, islet cells, and fibroblasts by the end of the third week. This was associated with proliferation of cells without zymogen granules (centroacinar, ductal, or undifferentiated?). These cells formed glandular structures which extended to the surface of the explant. By the end of the fourth week, explants resembled ductal hyperplasia with foci of carcinoma *in situ*. The distribution pattern of neoplasia in 343 explants examined after 10 weeks of DMN treatment was as follows: 79% resembled ductal cell carcinoma; 9%, ductal hyperplasia; and 3%, acinar cell carcinoma. Nude mice injected with cell suspensions prepared from 10-week-old culture developed subcutaneous nodules. These nodules resembled duct cell carcinoma with desmoplastic reaction. (Am J Pathol 84:469–478, 1976)

THE INCIDENCE OF PANCREATIC CARCINOMA in the United States has shown a considerable increase in recent years.[1-3] The etiologic agents in this type of cancer are not well defined, and until recently, no suitable model for study of carcinogenesis in this organ was available. In 1968, Druckery *et al.*[4] reported a low incidence of pancreatic adenocarcinoma in guinea pigs induced by methylnitrosourea (MNU) or methylnitrosourethane. Similar findings were described by Hiraki.[5] Since then, several investigators have reported the induction of pancreatic tumors by chemical carcinogens in a variety of animals.[6-11]

For the study of pancreas carcinogenesis in a model system with greater homogeneity in cell population and free of homeostatic mechanisms of the host, we have developed an *in vitro* model of carcinogenesis of rat pancreas. This report evaluates the histogenesis of tumor developed in rat pancreas organ culture, and after transplantation, the subsequent growth of these tumor cells in nude mice.

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Materials and Methods

Organ Culture

Methods for long-term culture of embryonic rat pancreas were described previously.[12] In summary, the pancreas anlagen of rat embryos were removed on the 13th day of gestation and cultured on a platform of Millipore filter, floating on a synthetic medium in roller vessels. The composition of the medium has been described previously.[13] The roller vessels were rotated at 0.5 rev/min, and tissues were incubated at 37 C in an atmosphere of 10% CO_2 in air saturated with water vapor. The medium was changed twice a week.

Dimethynitrosamine Treatment

Two days after the initiation of culture, the explants were transferred to roller vessels containing 5 ml of medium to which 100 μg of DMN (K & K Laboratories, Inc., New York, N.Y.) was freshly added. When the medium was changed, the replacement medium contained the same amount of DMN.

Microscopic Preparation

Twenty explants from each culture day, 1 through 14, and 10 explants from each culture week, 3 through 10, were examined by light microscope. The remaining explants (342) were examined after 10 weeks of treatment with DMN. Twenty of these explants were examined by electron microscope. For light microscopy, explants were fixed in Bouin's fluid, embedded in paraffin, and cut at 2 to 4 μ thickness. Sections were stained with hematoxylin and eosin.[14] For electron microscopy, specimens were fixed in 3% glutaraldehyde in sodium cacodylate buffered at pH 7.3,[15] followed by postosmication, dehydration in ethanol, and embedding in Epon 812.[16]

In Vivo Inoculation

Ten explants of cultures treated with DMN for 10 weeks were pooled and trypsinized by 0.05% solution of trypsin (Difco, 1 : 250) in aqueous saline (NaCl, 8 g/liter; $NaHCO_3$, 0.33 g/liter; KCl, 0.4 g/liter; and D-glucose, 1 g/liter) for 10 minutes at 37 C. After three washes with saline solution, cells were counted, and the concentration adjusted to 10^7 cells/ml. Similarly, a cell suspension of 10^7/ml was also prepared from control explants (grown for 10 weeks in the absence of DMN). Nude mice (courtesy of Dr. Tanapatchayapong), 5 to 7 weeks old, were injected subcutaneously in the dorsal aspect of the thorax with 0.1 ml of the cell suspensions prepared from DMN-treated or control explants.

Results

Morphologic Alterations

There was no immediate histologic change due to DMN administration. The histogenesis and differentiation of the pancreas rudiment occurred as previously described,[12,17] i.e., interconnecting tubular structures appeared on Day 4 of culture and acini lined by zymogen-containing acinar cells appeared by Day 5 of culture. On this day (third day of treatment) foci of acinar cell necrosis and degeneration were noticeable throughout the explants. Concomitantly, there were occasional mitotic figures in the centroacinar region of the necrotic acini. As the acinar cell degeneration and necrosis progressed during the next 10 days of culture

there also occurred an increase in cells which seemed to be of centroacinar, ductular, or undifferentiated cell type (Figure 1). By the second week of treatment the acinar cell necrosis was extensive and diffuse. In between the nuclear debris and degenerated acinar cells there were clusters of hypertrophic cells, with regular nuclei and large amounts of cytoplasm,forming irregular interconnecting tubules. These cells were devoid of zymogen granules and often presented a large apical cytoplasm. Mitotic figures were conspicuous (Figure 2). By the end of the third culture week, the explants were mainly composed of glandular structures lined with one or more layers of tall columnar cells showing numerous mitotic figures. Degenerated acinar cells with extreme vacuolization filled the space between the glands (Figure 3). Fibroblastic support was absent, and glands seemed to extend to the explant surface. Although many glands had polarity with peripherally located nuclei, foci of cribriform pattern, loss of polarity, and abnormal nuclei were noticeable.

The histologic appearance of explants studied beyond 3 weeks of treatment was variable. The degree of anaplasia, differentiation, and sometimes necrosis varied extensively. At one extreme was appearance of cystic papillary hyperplasia with atypical cells and secondary glandular formation (Figure 4). On the other extreme, there was appearance of explants composed of a solid mass of anaplastic cells with little cytoplasm, foci of necrosis, and only occasional areas with some degree of differentiation (Figure 5). Most explants studied revealed a mixture of these two extremes, i.e., foci of anaplastic cells intermingled with areas of apparent cystic papillary adenocarcinoma. Mucus production as revealed by periodic acid–Schiff stain and electron microscope observation was irregularly distributed and was even absent in some cells.

Exocrine Tumor Classification

In the evaluation of 342 explants treated for 10 weeks with DMN we have used the criteria described by Cubilla and Fitzgerald [18] for evaluating human pancreatic carcinoma. The results are shown in Table 1. Twenty-nine explants were necrotic and not included. Lesions resembling

Table 1—The Distribution Pattern of Neoplasia in Pancreas Organ Culture According to Similarity to Human Pancreatic Carcinoma

Week of treatment	Resembling ductal hyperplasia with *in situ* carcinoma	Resembling duct cell carcinoma	Resembling acinar cell carcinoma
4-9	18	39	—
10	32	269	10

in situ carcinomas in ductal hyperplasia were more frequent between 4 and 6 weeks of DMN treatment. The main histologic pattern found resembled that of duct cell carcinoma with various degrees of anaplasia (79%). A small number (3%) were similar to acinar cell carcinomas. These were highly cellular, with many undifferentiated cells, occasional zymogen-containing cells, and only a few acini (Figure 6). As revealed by the electron microscope, zymogen granules were absent from many cells, and when present, they were of irregular size and shape (Figure 7). Cylindrically shaped granules were not uncommon. Condensing vacuoles (*prozymogen*) were not common; instead, electron-dense material was often present in dilated cisternae surrounded by rough endoplasmic reticulum.

Endocrine Tumor

Islet cells or their remnants were seen up to the second week of culture. They were not observed in 3 weeks culture and thereafter; however, two explants of 402 studied revealed a single mass of islet cells some with atypical nuclei.

In Vivo Growth in Nude Mice

Controls

A careful search of serial sections taken from subcutaneous injection sites in mice 6 months after inoculation of cells derived from control explants revealed no sign of injected cells.

DMN Treated Mice

As early as 2 weeks after injection of cells derived from DMN-treated explants, multiple small 1 to 2 mm subcutaneous nodules were palpable at the site of injection. These nodules increased in size and number and, by the second month, were not only palpable but also had become visible as a small elevation of the skin.

Histologically, they ranged from cell aggregates in early phase to well-formed nodules composed of solid cords and glands with irregular lumens (Figures 8 and 9). Cells with abnormal nuclei and variable amounts of cytoplasm in different shapes and sizes were present throughout the sections. Severe desmoplastic reaction and foci of necrosis were seen. No metastatic lesions were identified.

Discussion

The present data confirm the earlier report [19] that DMN induced pancreatic neoplasms in organ culture within 6 to 10 weeks. The majority of

tumors (79%) appeared as duct cell carcinomas. The slow growth of these tumors in nude mice compares with the linear growth of human adenocarcinoma of pancreas reported by Schmidt and Good.[20] They reported 5 of 13, or a "take" of 38.4%; however, we had a 100% "take"—6 of 6 inoculations. This difference in "percent take" may be explained by the fact that we have used single cell suspension as opposed to tissue fragments used by these investigators.

Recent efforts leading to the chemical induction of carcinoma of the pancreas in experimental animals have been moderately successful.[7-11] Longnecker and Curphey have reported predominantly acinar cell carcinoma in rats induced by azaserine.[7] Reddy *et al.*[10] and Reddy and Rao [11] have described pancreatic tumors induced by MNU in guinea pigs. Pour *et al.*[8,9] have observed predominantly duct cell carcinoma in Syrian golden hamsters treated with 2,2'-dihydroxy-di-N-propylnitrosamine with an average latency of 26 to 27 weeks.

The present model of *in vitro* induction of pancreatic carcinoma has a number of features which makes it superior to some of the animal models. Thus, the chemical effects are seen in a relatively short time (e.g., 10 weeks or less) with a high degree of certainty and with very modest amounts of carcinogen. The distribution pattern of these tumors is similar to that described in human pancreatic carcinoma,[18] namely that it has a high incidence of duct cell carcinoma.

The model allows for certain studies to be done with relative ease. Thus, it could be easily used for purposes of screening of possible carcinogens, to determine degree of carcinogenicity. The use of a chemically defined medium should make it possible to detect macromolecular secretory changes. Finally, the absence of connective tissue and normal epithelium in this system makes it a desirable model for the study of different stages (reversible and nonreversible) of carcinogenesis, i.e., it would be possible to study and characterize ultrastructurally and at the biochemical level the progenitor cells for adenocarcinoma of the pancreas.

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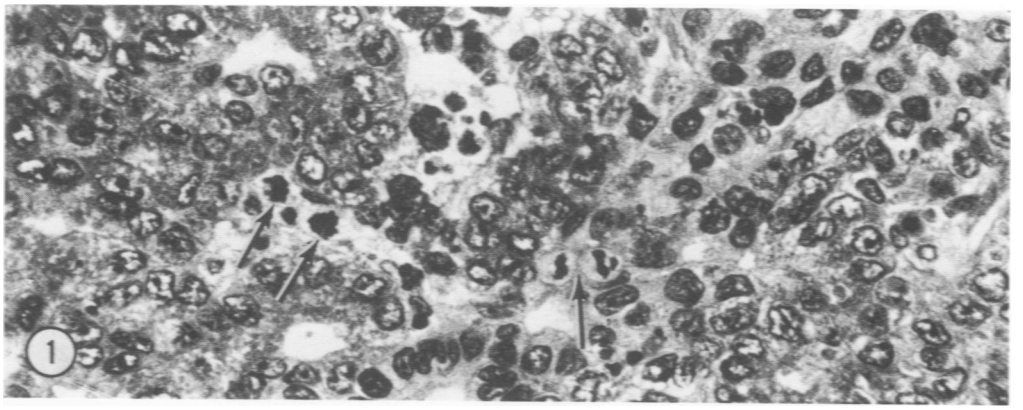
Legends for Figures

Figures 1–6, 8 and 9 are light microscopy sections, fixed in Bouin's fixative, embedded in paraffin, and stained with hematoxylin and eosin.

Figure 1—Pancreas rudiment removed from Day 13 embryo and organ cultured for 9 days (7 days with DMN). Foci of acinar cell degeneration and necrosis are seen. Mitoses in the centroacinar and ductular regions are shown (*arrows*). (× 560)

Figure 2—Pancreas rudiment cultured for 16 days (2 weeks with DMN) showing massive necrosis and degeneration of acinar cells and clusters of hypertrophic epithelial cells with large amount of cytoplasm, regular nuclei, and irregular glandular structures. (× 580)

Figure 3—Pancreas anlage cultured for 23 days (3 weeks with DMN). Proliferation of glandular structures lined by one or more layers of epithelium, some with normal nuclei (variation in size, shape and staining), is shown. Stroma is virtually absent, and proliferating epithelium is seen at the periphery of explants without fibroblasts. Remnants of degenerating acinar cells are present. (× 160)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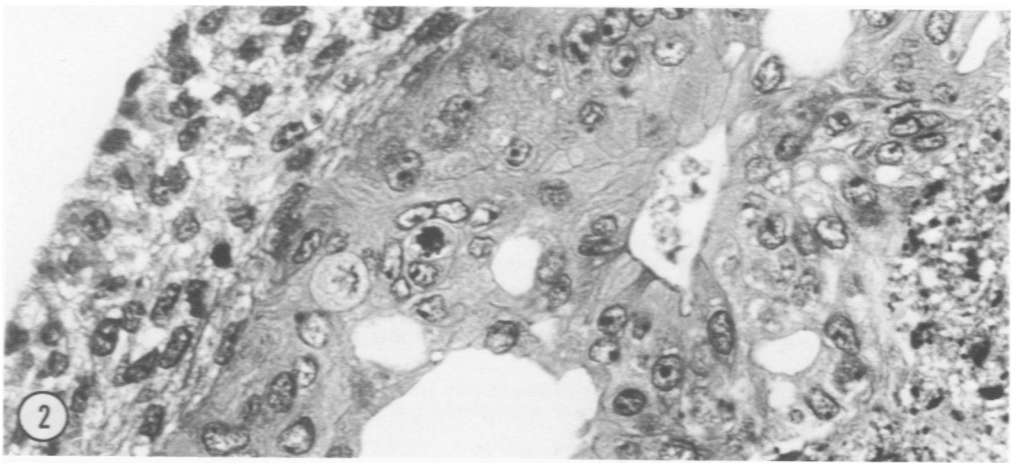
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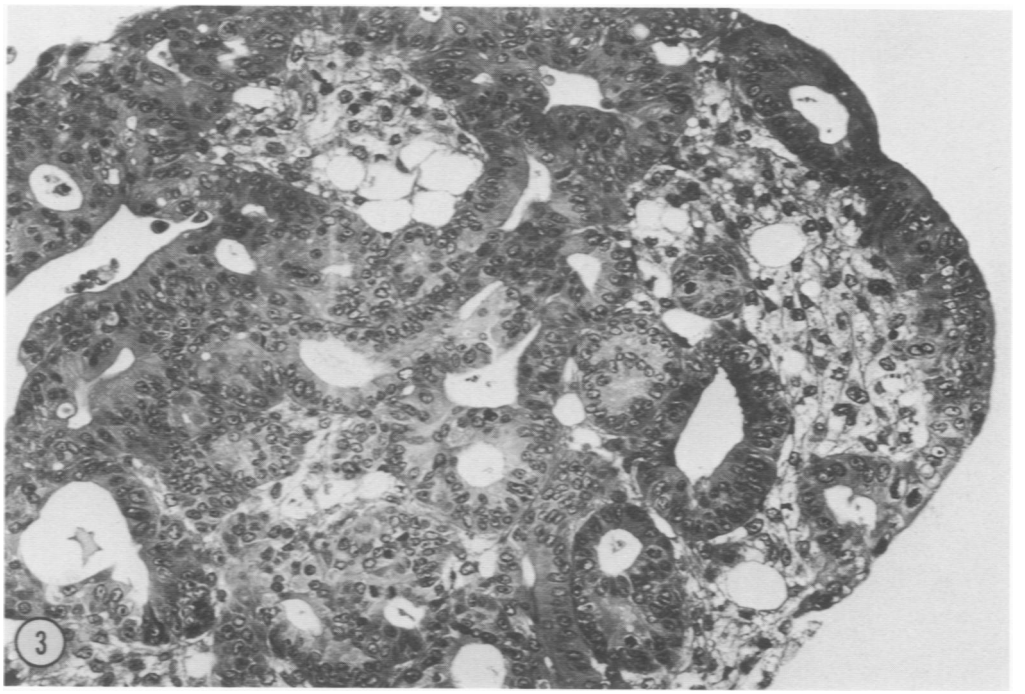
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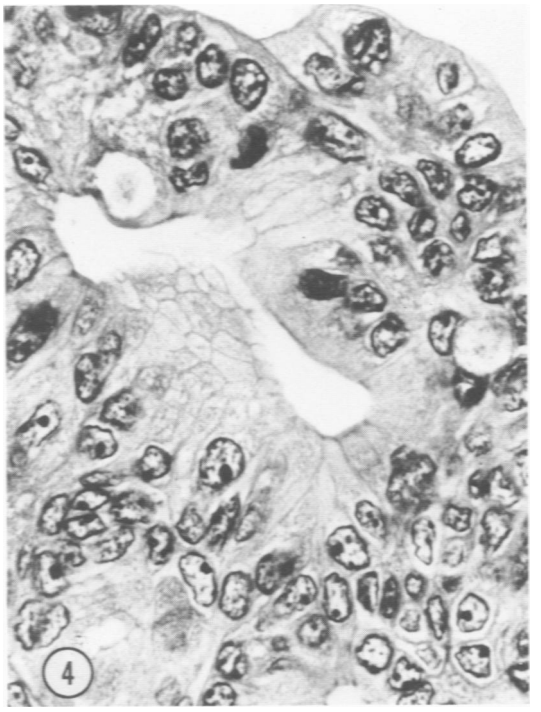
Figure 4—Pancreas rudiment cultured for 3 weeks in the presence of DMN, resembling papillary carcinoma with secondary lumens (cribriform pattern). (× 500)

Figure 5—Pancreas rudiment cultured for 6 weeks with DMN. Bizarre tumor cells showing large, irregular dark nuclei with various amounts of cytoplasm. (× 500)

Figure 6—Pancreas anlage cultured for 10 weeks with DMN, resembling acinar cell carcinoma. Cells with various amount of cytoplasm and abnormal, peripherally located nuclei. Few acinar structures are seen. (× 200)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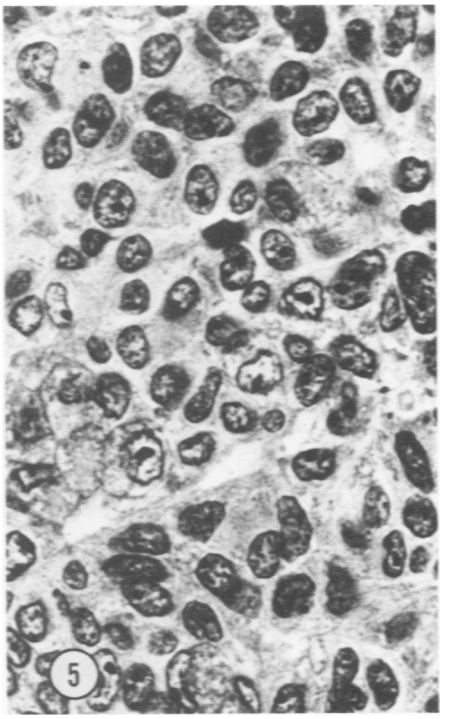
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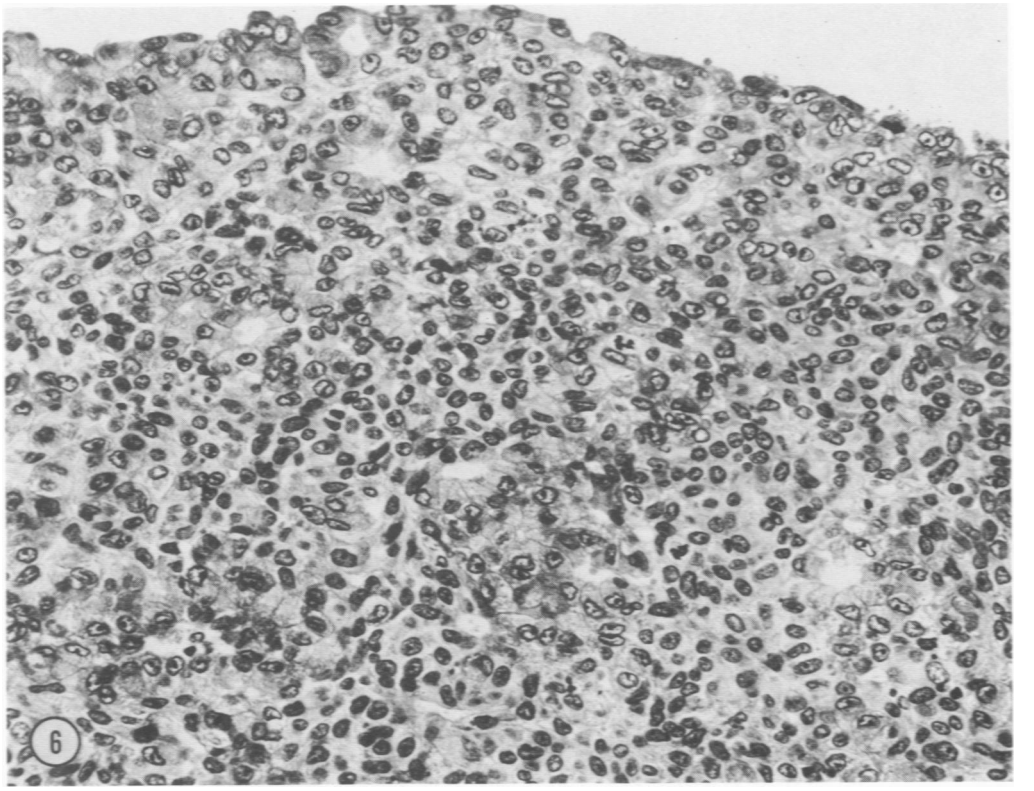
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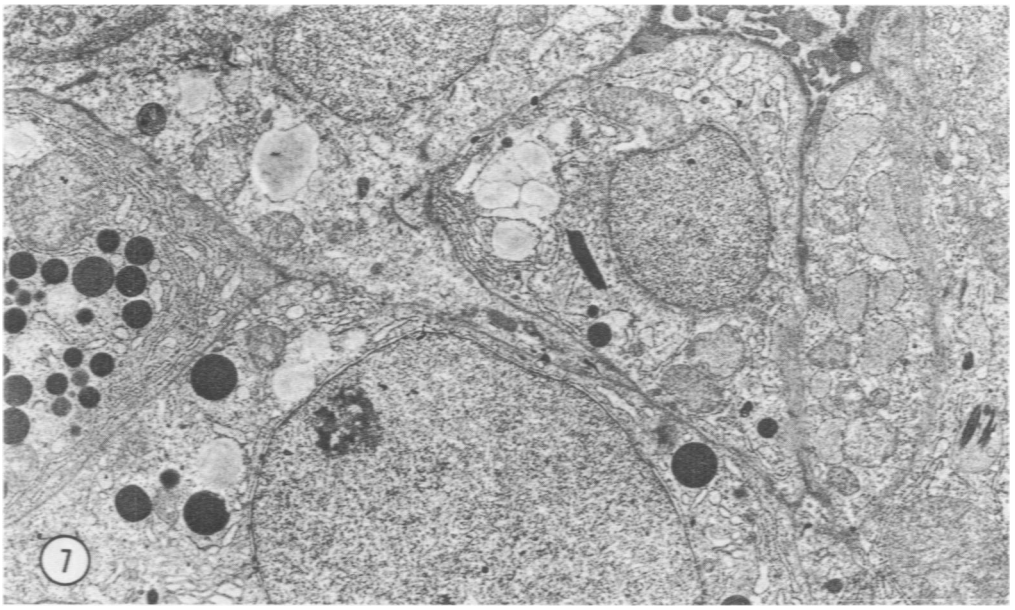

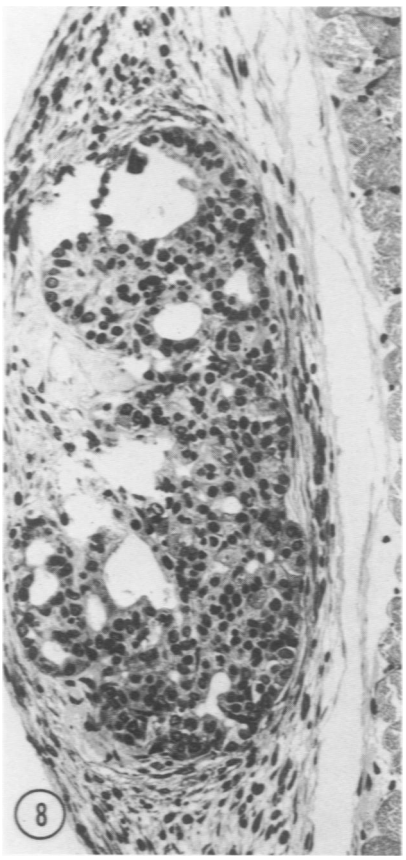

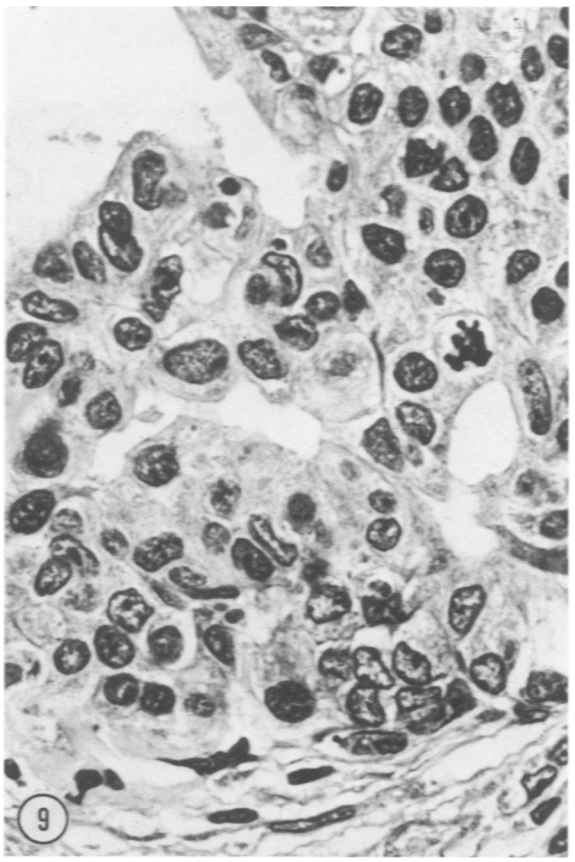

Figure 7—Pancreas anlage cultured for 10 weeks with DMN, fixed in glutaraldehyde, postosmicated, embedded in Epon, and stained with lead hydroxide. Zymogen granules of various shapes and sizes are seen. Electron-dense material in dilated cisternae are present. (× 7000) **Figures 8 and 9** are light microscopic sections of nodules developed in nude mice 5 weeks after subcutaneous injections of 10^6 cells prepared from pancreas culture for 10 weeks in the presence of DMN in organ culture. **Figure 8**—A nodule developed 5 weeks after the injection of cell suspension; it resembles duct cell carcinoma associated with desmoplastic reaction. (× 82) **Figure 9**—Micrograph showing abnormal mitotic figures and a mixture of solid and glandular patterns seen in the original tumor. (× 580)